

Cell Wall β -(1,6)-Glucan of *Saccharomyces cerevisiae*

STRUCTURAL CHARACTERIZATION AND IN SITU SYNTHESIS^{*[§]}

Received for publication, October 3, 2008, and in revised form, March 11, 2009. Published, JBC Papers in Press, March 11, 2009, DOI 10.1074/jbc.M807667200

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Despite its essential role in the yeast cell wall, the exact composition of the β -(1,6)-glucan component is not well characterized. While solubilizing the cell wall alkali-insoluble fraction from a wild type strain of *Saccharomyces cerevisiae* using a recombinant β -(1,3)-glucanase followed by chromatographic characterization of the digest on an anion exchange column, we observed a soluble polymer that eluted at the end of the solvent gradient run. Further characterization indicated this soluble polymer to have a molecular mass of \sim 38 kDa and could be hydrolyzed only by β -(1,6)-glucanase. Gas chromatography-mass spectrometry and NMR (¹H and ¹³C) analyses confirmed it to be a β -(1,6)-glucan polymer with, on average, branching at every fifth residue with one or two β -(1,3)-linked glucose units in the side chain. This polymer peak was significantly reduced in the corresponding digests from mutants of the *kre* genes (*kre9* and *kre5*) that are known to play a crucial role in the β -(1,6)-glucan biosynthesis. In the current study, we have developed a biochemical assay wherein incubation of UDP-[¹⁴C]glucose with permeabilized *S. cerevisiae* yeasts resulted in the synthesis of a polymer chemically identical to the branched β -(1,6)-glucan isolated from the cell wall. Using this assay, parameters essential for β -(1,6)-glucan synthetic activity were defined.

The cell wall of *Saccharomyces cerevisiae* and other yeasts contains two types of β -glucans. In the former yeast, branched β -(1,3)-glucan accounts for \sim 50–55%, whereas β -(1,6)-glucan represents 10–15% of the total yeast cell wall polysaccharides, each chain of the latter extending up to 140–350 glucose residues in length. The amount of 3,6-branched glucose residues varies with the yeast species: 7, 15, and 75% in *S. cerevisiae*, *Candida albicans*, and *Schizosaccharomyces pombe*, respectively (1). β -(1,6)-Glucan stabilizes the cell wall, since it plays a central role as a linker for specific cell wall components, including β -(1,3)-glucan, chitin, and mannoproteins (2, 3). However, the exact structure of the β -(1,6)-glucan and the mode of biosynthesis of this polymer are largely unknown. In *S. pombe*, immunodetection studies suggested that synthesis of this polymer backbone begins in the endoplasmic reticulum, with exten-

sion occurring in the Golgi (4) and final processing at the plasma membrane. In *S. cerevisiae*, Montijn and co-workers (5), by immunogold labeling, detected β -(1,6)-glucan at the plasma membrane, suggesting that the synthesis takes place largely at the cell surface.

More than 20 genes, including the *KRE* gene family (14 members) and their homologues, *SKN1* and *KNH1*, have been reported to be involved in β -(1,6)-glucan synthesis in *S. cerevisiae*, *C. albicans*, and *Candida glabrata* (6–10). Among all of these genes, the ones that seem to play the major synthetic role are *KRE5* and *KRE9*, since their disruption caused significant reduction (100 and 80%, respectively, relative to wild type) in the cell wall β -(1,6)-glucan content (11–13).

To date, the biochemical reaction responsible for the synthesis of β -(1,6)-glucan and the product synthesized remained unknown. Indeed, in most cases, when membrane preparations are incubated with UDP-glucose, only linear β -(1,3)-glucan polymers are produced, although some studies have reported the production of low amounts of β -(1,6)-glucans by membrane preparations (14–17). These data suggest that disruption of the fungal cell prevents or at least has a strong negative effect on β -(1,6)-glucan synthesis. The use of permeabilized cells, which allows substrates, such as nucleotide sugar precursors, to be readily transported across the plasma membrane, is an alternative method to study *in situ* cell wall enzyme activities (18–22). A number of methods have been developed to permeabilize the yeast cell wall (23), of which osmotic shock was successfully used to demonstrate β -(1,3)-glucan and chitin synthase activities (20, 24). Herein, we describe the biochemical activity responsible for β -(1,6)-glucan synthesis using permeabilized *S. cerevisiae* cells and UDP-[¹⁴C]glucose as a substrate. We also have analyzed the physicochemical parameters of this activity and chemically characterized the end product and its structural organization within the mature yeast cell wall.

EXPERIMENTAL PROCEDURES

Yeast Strain and Growth Conditions—*S. cerevisiae* strains used were BY4741 (EUROSCARF, kindly provided by Unité de Génétique Moléculaire des Levures, Institut Pasteur, Paris, France), Δ *kre9*/YJL174W (heterozygous diploid) (11), *kre5-ts2* (kindly provided by Dr. Guillaume Lesage, Cell Imaging and Analysis Network, Department of Biology, McGill University, Montreal, Canada) (25), and YG0091 (*Mat α Δ alg5::HIS3 *ade2-101 his3-200 ura3-52*) (kindly provided by Dr. Markus Aebi, Institute of Microbiology, ETH Zürich, Switzerland) (26). Haploid Δ *kre9* (*MAT α Δ kre9::HIS3*) was obtained by tetrad dissec-*

^{*} This work was supported in part by European Union STREP Fungwall Grant LSHB CT 2004-511952.

^[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1–3.

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tion from the diploid strain $\Delta kre9/YJL174W$ (11). Cells were grown in YPD (2% glucose, 1% peptone, and 2% yeast extract) at 30 °C and harvested in their early logarithmic phase of growth ($A_{600} = 2-3$). *kre5-ts2* was grown initially at 25 °C on YPD agar, following which 1×10^6 cells were inoculated into liquid YPD and then grown at the restrictive temperature (37 °C) and harvested in their early logarithmic growth phase.

Recovery of an Alkali-insoluble (AI)³ Fraction from the Cell Walls—Cells were washed twice with water and boiled in 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM EDTA, 2% SDS, and 40 mM β -mercaptoethanol (β -ME) for 1 h twice. The sediment obtained after centrifugation ($3000 \times g$, 10 min) was washed five times with water and then incubated in 1 M NaOH containing 0.5 M NaBH₄ at 65 °C for 1 h twice. The insoluble pellet obtained upon centrifugation of this alkali-treated sample ($3000 \times g$, 10 min, AI fraction) was washed with water to neutrality and freeze-dried.

Enzymatic Treatments of AI Fraction—The AI fraction was incubated sequentially either with an endo- β -(1,3)-glucanase and then an endo- β -(1,6)-glucanase or with an endo- β -(1,6)-glucanase and then an endo- β -(1,3)-glucanase. LamA (endo- β -(1,3)-glucanase) was a recombinant protein from *Thermotoga neapolitana* expressed in *Escherichia coli* (a kind gift from Dr. Vladimir V. Zverlov, Institute of Molecular Genetics, Russian Academy of Science, Moscow, Russia) (27). Endo- β -(1,6)-glucanase from *Trichoderma harzianum* was expressed in *Pichia pastoris* (a kind gift from Prof. Stanley Brul, Swammerdam Institute for Life Sciences, Amsterdam, The Netherlands) (28). Digestion was undertaken by treating 1 mg of AI fraction with 2 units (μ mol of reducing equivalents released/h/mg of protein) of the endo- β -(1,3)-glucanase in acetate buffer (50 mM, pH 6.2) at 37 °C for 60 h. Hydrolysis with endo- β -(1,6)-glucanase was performed by incubating 1 mg of sample (in acetate buffer; 50 mM, pH 5.5) with 2.5 units of enzyme at 37 °C for 24 h. Preliminary kinetic assays demonstrated that the incubation time and enzyme concentration were critical to the optimal digestion of the respective polymers, since the addition of more enzyme or a longer incubation time for the assay did not result in the release of additional reducing carbohydrates.

HPLC Methods—The enzyme digests (the supernatants obtained after centrifugation of the reaction mixture at $10,000 \times g$ for 10 min) were analyzed by high performance anion exchange chromatography on a CarboPAC PA-1 column (4.6×250 mm) using NaOH (50 mM) and NaOAc (500 mM in 50 mM NaOH) as solvent A and B, respectively. The column was pre-equilibrated for 20 min with 98% A + 2% B. Following sample injection, a gradient run (flow rate 1 ml/min) was performed as follows: 0–2 min, isocratic step (98% A + 2% B), 2–15 min 98% A + 2% B to 65% A + 35% B, 15–22 min 65% A + 35% B to 57% A + 43% B, 22–23 min 57% A + 43% B to 100% B, and

23–25 min 100% B. Samples were detected on a pulsed electrochemical detector (nonradiolabeled digests) or using a radiometric detector (Packard Radiomatic Flo-one, equipped with a 500- μ l liquid type cell) for the ¹⁴C-radiolabeled samples. Specific detection of the ¹⁴C-radiolabeled compounds was performed at 156 keV with a scintillant at a flow rate of 2.0 ml/min.

Low Pressure Liquid Chromatography—Soluble samples were subjected to gel filtration on a TSK HW40S column (90×1.4 cm; Toso-Pore), using 0.5% (v/v) acetic acid as the eluant at a flow rate of 0.2 ml/min. The carbohydrate-containing fractions were identified with a refractive index detector (Gilson) and also by the phenol-sulfuric method. The average M_r of the polymer was determined using a Sephadex-G75 column (90×1.4 cm; Amersham Biosciences) with 0.5% acetic acid as the eluant at a flow rate of 12 ml/h. A calibration curve was obtained by plotting $\log M_r$ of dextran standards (Amersham Biosciences) against their V_e/V_o , where V_e is the elution volume of the sample and V_o is the column void volume.

Gas Chromatography (GC)/Gas-Liquid Chromatography (GLC)-Mass Spectrometry (MS)—Vacuum-dried samples (2 mg) were subjected to methylation using the lithium methyl sulfinyl carbanion procedure (29) modified by Fontaine *et al.* (30). Then alditol acetates were obtained after hydrolysis (4 N trifluoroacetic acid, 100 °C, 4 h), reduction, and peracetylation of the methylated samples (31). GLC-MS analysis was recorded using an Automass II 30 quadrupole mass spectrometer interfaced with a Carlo Erba 8000 Top gas chromatograph (Finnigan, Argenteuil, France). Electron ionization spectra were recorded using ionization energy of 70 eV. The gas chromatograph was equipped with a TR-1 MS capillary column ($30 \text{ m} \times 0.25 \text{ mm}$; Chrompack), and helium was the mobile phase used at a flow rate of 1.2 ml/min; the column temperature was raised from 100 to 240 °C at a rate of 5 °C/min.

NMR Analyses—NMR spectra were acquired at 308 K on a Varian spectrometer operating at a proton frequency of 500 MHz and equipped with a triple resonance ¹H {¹³C/¹⁵N} triax probe (Varian, Les Ulis, France). Samples were dissolved in 420 μ l of D₂O (99.97% ²H atoms; Euriso-top; Commissariat à l'Energie Atomique, Saclay, France) and transferred in a 5-mm Shigemi tube (Shigemi Inc., Alison Park, PA). ¹H chemical shifts were referenced to external 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (its methyl resonance was set to 0 ppm). ¹³C chemical shifts were then calculated from ¹H chemical shifts and γ ratio relative to DSS. A ¹³C/¹H γ ratio of 0.251449530 was used (32).

The following nuclei assignment strategy was adopted. First, the proton resonances were assigned using two-dimensional COSY and RELAY experiments with one and two relays of 60 ms in order to follow connectivities from the anomeric proton up to the H4 proton of the glycosidic residues (33, 34). The intraglycosidic residue spin systems were completed by means of a TOCSY experiment with a long mixing time (100 ms) (35). A ¹H-¹³C edited gHSQC experiment and a ¹H-¹³C gHSQC-TOCSY experiment with a mixing time of 80 ms was used to achieve ¹³C chemical shift assignments from previously identified ¹H resonances (36). Meanwhile, the CH₂ groups were easily identified from the ¹H-¹³C-edited gHSQC experiment. Then

³ The abbreviations used are: AI, alkali-insoluble; COSY, correlation spectroscopy; gHMBC, gradient-selected heteronuclear multiple bond correlation; gHSQC, gradient-selected heteronuclear single-quantum correlation; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; β -ME, β -mercaptoethanol; HPLC, high pressure liquid chromatography; GC, gas chromatography; GLC, gas-liquid chromatography; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; DP, degree of polymerization.

^1H , ^1H coupling constant analysis from one-dimensional and/or COSY spectrum (^1H resolution of 0.15 Hz and 1.5 Hz respectively) assessed the identity of monosaccharides. Moreover, the anomeric configuration of monosaccharide residues was established from knowledge of $^3J_{1,2}$ values and confirmed by the measurement of the $^1J_{\text{C1H1}}$ heteronuclear coupling constants in the ^1H dimension of the gHMBC spectrum (^1H resolution of 0.5 Hz) (36). Finally, glycosidic linkages were established via through-space dipolar interactions using ^1H - ^1H NOESY experiments (mixing time of 200 ms) (37) and/or via three-bond interglycosidic ^1H - ^{13}C correlations using a ^1H - ^{13}C gHMBC experiment (long range delay of 60 ms).

Cell Permeabilization—The method of Crotti *et al.* was used (20). Briefly, 10^{10} cells were shaken with 0.04 M EDTA (10.5 ml) and β -ME (14.3 M, 72 μl) at 30 °C for 1 h and recovered by centrifugation ($3300 \times g$, 10 min). The yeast pellet was washed with 0.8 M sorbitol (15 ml) and shaken again in citrate-phosphate buffer, pH 6.2 (20.2 ml) containing EDTA (1 mM) and sorbitol (0.8 M) at 30 °C for 1 h. After centrifugation, the cells were subjected to osmotic shock by suspending them in 15 ml of cold 50 mM Tris-HCl, pH 7.5, on ice for 15 min. Finally, 10^{10} cells were suspended in 0.05 M Tris-HCl (pH 7.5, 1 ml). The efficacy of the protocol was checked by plating 50 μl of the cell suspension on YPD agar.

Cell Fraction Preparation—Intact as well as permeabilized cells were washed twice with 50 mM Tris-HCl, pH 7.5, suspended in the same buffer (10^{10} cells in 4 ml of buffer), and subsequently broken with glass beads (0.5 mm) in FastPrep (MP Biomedicals) by four cycles of vortex for 30 s interspersed with 1-min incubations on ice. Cells were also disrupted with glass beads (0.5 mm) in an MSK Braun cell homogenizer under a CO_2 cooling system. The cell wall fraction was removed by low speed centrifugation ($3000 \times g$, 5 min at 4 °C) of the broken cells. The supernatant was subjected to a second centrifugation at $35,000 \times g$ for 30 min at 4 °C, and the pellet obtained (membrane fraction) was suspended in 50 mM Tris-HCl (pH 7.5) containing 33% (v/v) glycerol (17), whereas the supernatant served as intracellular fraction. Protein content of the extracts was measured with the Bradford reagent (Bio-Rad), using bovine serum albumin as a standard. All of the fractions were stored at -70 °C or directly used for enzymatic assays.

β -(1,6)-Glucan Synthase Assay—The assay mixture, in a total volume of 67 μl , contained 50 mM Tris-HCl buffer (pH 7.5), 0.5 mM UDP- ^{14}C glucose (specific activity 34 nmol/125 nCi in the final reaction mixture), 0.2 mM ATP, 20 μM GTP γS , 0.2 mM MgCl_2 , 20 mM *N*-acetylglucosamine, and membrane fraction (100 μg of protein) or permeabilized cells (5×10^8 cells, equivalent to 27 μg of protein of membrane fraction). Neosynthesized polysaccharides were precipitated by two volumes of cold ethanol (-20 °C). The precipitate thus obtained was washed three times with water (500 μl each) and treated with 500 μl of 1 N NaOH containing 0.5 M NaBH_4 at 65 °C for 1 h twice. The AI fraction was collected by centrifugation at $15,000 \times g$ for 10 min, washed with water, neutralized using acetic acid, and characterized further by sequential enzymatic treatments (either with LamA and then an endo- β -(1,6)-glucanase or with an endo- β -(1,6)-glucanase and then LamA), HPLC, and methylation analysis as described above. Incorporation of the radioac-

tivity was measured at each step using a Wallac 1410 liquid scintillation counter (PerkinElmer Life Sciences). Results obtained in cpm were converted into nmol of glucose (as per the instructions from Amersham Biosciences). Specific enzymatic activity was calculated per μg of membrane protein equivalent (obtained from the same cell counts). Data are presented as means \pm S.E. Single group comparisons were analyzed with Student's *t* test. Statistical significance was set as $p < 0.05$ for significant.

TLC—Analysis of the methylated radiolabeled polymer was performed after trifluoroacetic acid (4 N, 100 °C, 4 h) hydrolysis. The hydrolysate was dried under vacuum and reconstituted in methanol (50 μl). Aliquots of 5 μl (corresponding to 1500 cpm) were spotted on an aluminum-backed Silica Gel-60 plate (Merck). Samples were separated using acetonitrile/chloroform/methanol (3:9:1) as the mobile phase (two ascents) (38).

The gentio-oligosaccharides obtained from radiolabeled 23.6 min polymer upon endo- β -(1,6)-glucanase digestion were separated on a BIOGEL-P2 column (130×1.5 cm, flow rate 8 ml/h, using 0.5% acetic acid as the solvent). The oligomeric fraction was dried under vacuum and reconstituted in water (10 μl), and 5- μl aliquots (corresponding to 500 cpm) were spotted on an aluminum-backed Silica Gel-60 plate (Merck). The migration was performed using propanol/acetone/water (9:6:5) as the mobile phase (two ascents). All of the plates were revealed using orcinol- H_2SO_4 reagent. Autoradiograms were obtained by exposing TLC plates to a storage phosphor screen placed inside an exposure cassette and developed by using a PhosphorImager (STORM 850; Amersham Biosciences) using ImageQuant software (version 5.2).

Periodate Oxidation/Smith Degradation (39)—Five mg (dry weight) samples were incubated in 2 ml of sodium *meta*-periodate, 100 mM, during for days at 4 °C in the dark. Excess periodate was destroyed following the addition of 400 μl of ethylene glycol. After dialysis against water (membrane cut-off, 1000 Da), the oxidized product was reduced for 2 h in 100 mM ammonium hydroxide (2 ml) containing 20 mg of NaBH_4 . Excess reagent was removed upon repeated co-distillations with methanol. Smith degradation was performed with 10% acetic acid at 100 °C for 1 h. Products were freeze-dried, reconstituted in water, and analyzed on a CarboPAC PA-1 chromatography column.

Matrix-assisted Desorption Ionization/Time of Flight (MALDI-TOF) MS—Spectra were acquired on a Voyager Elite DE-STR mass spectrometer (Perspective Biosystems, Framingham, MA) equipped with a pulsed nitrogen laser (337 nm) and a gridless delayed extraction ion source. The spectrometer was operated in positive reflectron mode by delayed extraction with an accelerating voltage of 20 kV, a pulse delay time of 200 ns, and a grid voltage of 66%. Samples were prepared by mixing directly on the target 0.5 μl of oligosaccharide solution in water (10–50 pmol) with 0.5 μl of 2,5-dihydroxybenzoic acid matrix solution (10 mg/ml in $\text{CH}_3\text{OH}/\text{H}_2\text{O}$, 50:50, v/v). The samples were allowed to dry for about 5 min at room temperature. Between 50 and 100 scans were averaged for every spectrum.

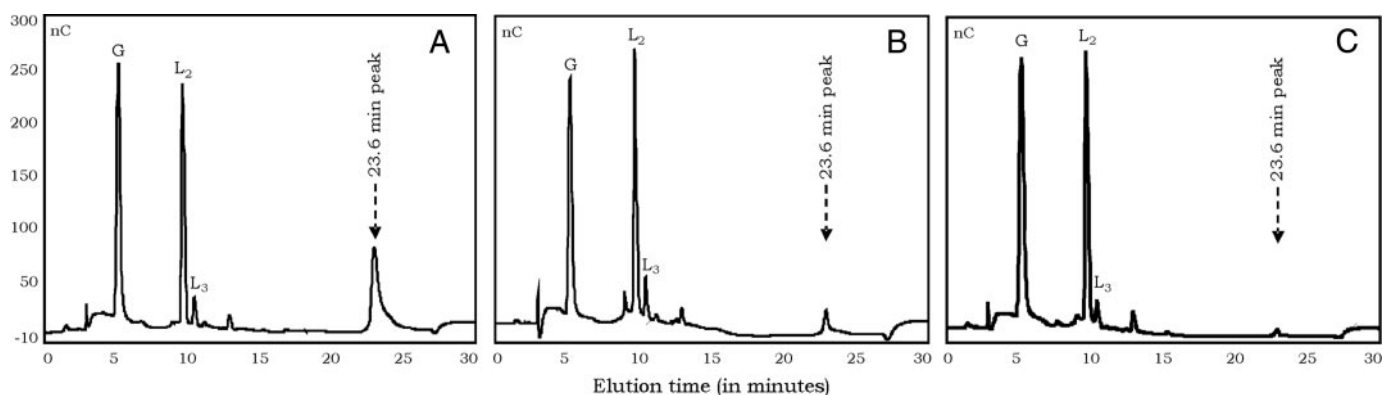


FIGURE 1. **Chromatography profiles of the endo- β -(1,3)-glucanase solubilized cell wall alkali-insoluble fractions (nonradiolabeled).** Chromatography was performed on CarboPAC PA-1 DIONEX column connected to a pulsed electrochemical detector. A, wild-type BY4741 strain; B, $\Delta kre9$; C, $kre5-ts2$ mutants. The peaks at 5.2, 9.7, and 10.4 min represent glucose (G), laminaribiose (L_2) and laminaritriose (L_3). The 23.6 min peak is marked by a broken arrow.

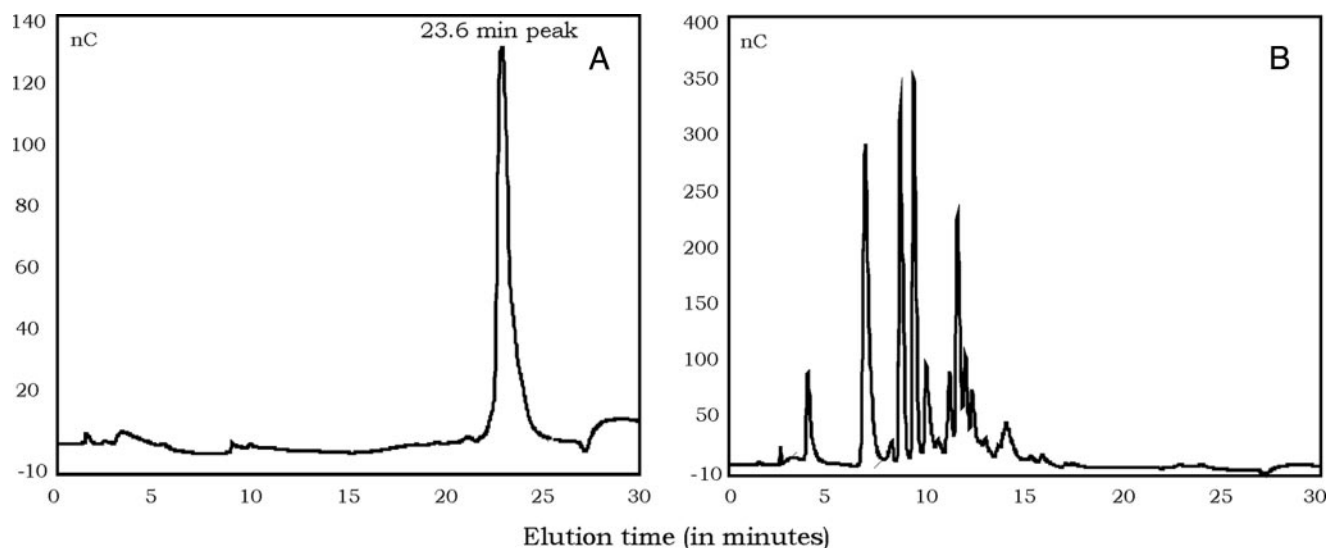


FIGURE 2. **Degradation pattern of the 23.6 min peak (purified on a TSK-HW40S column) digested by an endo- β -(1,6)-glucanase.** A, control; B, after 24 h of β -(1,6)-glucanase treatment. Chromatography conditions are as in Fig. 1.

RESULTS

Solubilization of AI Fraction Using Recombinant Endo- β -(1,3)-glucanase—Solubilization of the cell wall AI fraction from the wild-type *S. cerevisiae* strain (BY4741) using an endo- β -(1,3)-glucanase followed by anion exchange column chromatography of the digest resulted in a profile containing glucose and laminaribiose and laminaritriose (eluted at 5.2, 9.7, and 10.4 min, respectively). In addition, a soluble polymer was eluted from the column at the end (23.6 min) of the solvent gradient run (with 100% solvent B, designated as the “23.6 min fraction” hereafter). The endo- β -(1,3)-glucanase-solubilized AI fraction from *kre* mutants ($\Delta kre9$ and $kre5-ts2$) showed a significant diminution (90 and 98%, respectively, compared with BY4741) in this 23.6 min peak area (Fig. 1, B and C, respectively), suggesting that this peak could be rich in β -(1,6)-glucan.

Isolation and Structural Characterization of the 23.6 min Fraction—The polymer, eluting at 23.6 min from the anion exchange column, was isolated in bulk upon gel permeation chromatography of the endo- β -(1,3)-glucanase-solubilized cell wall AI fraction from the wild type strain of *S. cerevisiae* on a TSK HW40S column, wherein it eluted in the column void volume. The average molecular mass of this polymer that

migrated as a single peak on a Sephadex G75 column was calculated to be 38 ± 2 kDa. Alditol acetates obtained upon successive hydrolysis, NaBH_4 reduction, and acetylation of this gel permeation chromatography-purified polymer demonstrated the presence of only glucose upon GLC analysis. Further, the polymer upon methylation and GC-MS analysis showed mainly alditol acetates for 2,3,4-Me₃Glc ((1,6)-linked), followed by 2,3,4,6-Me₄Glc (permethylated) and 2,4-Me₂Glc (3,6-branched) species in an approximate ratio of 5:1.5:1.2 and a trace amount of 2,4,6-Me₃-Glc ((1,3)-linked) species. In addition, this polymer was resistant to endo- β -(1,3)-glucanase digestion but completely degraded by the recombinant endo- β -(1,6)-glucanase from *T. harzianum* (Fig. 2), which resulted in the release of glucose (eluted at 5.2 min from the anion exchange column) and an oligomeric mixture (eluted between 8.2 and 13.3 min) (Fig. 2). Purification of this mixture was undertaken on a TSK HW40S column, and the oligomers thus obtained were further characterized by MALDI-TOF-MS, GC-MS, and NMR. The peaks with retention times between 8.2 and 11.4 min on the CarboPac PA1 anion exchange column corresponded to that of the gentiobiose to gentiopentaose, standards, respectively, suggesting their linear structure. Fur-

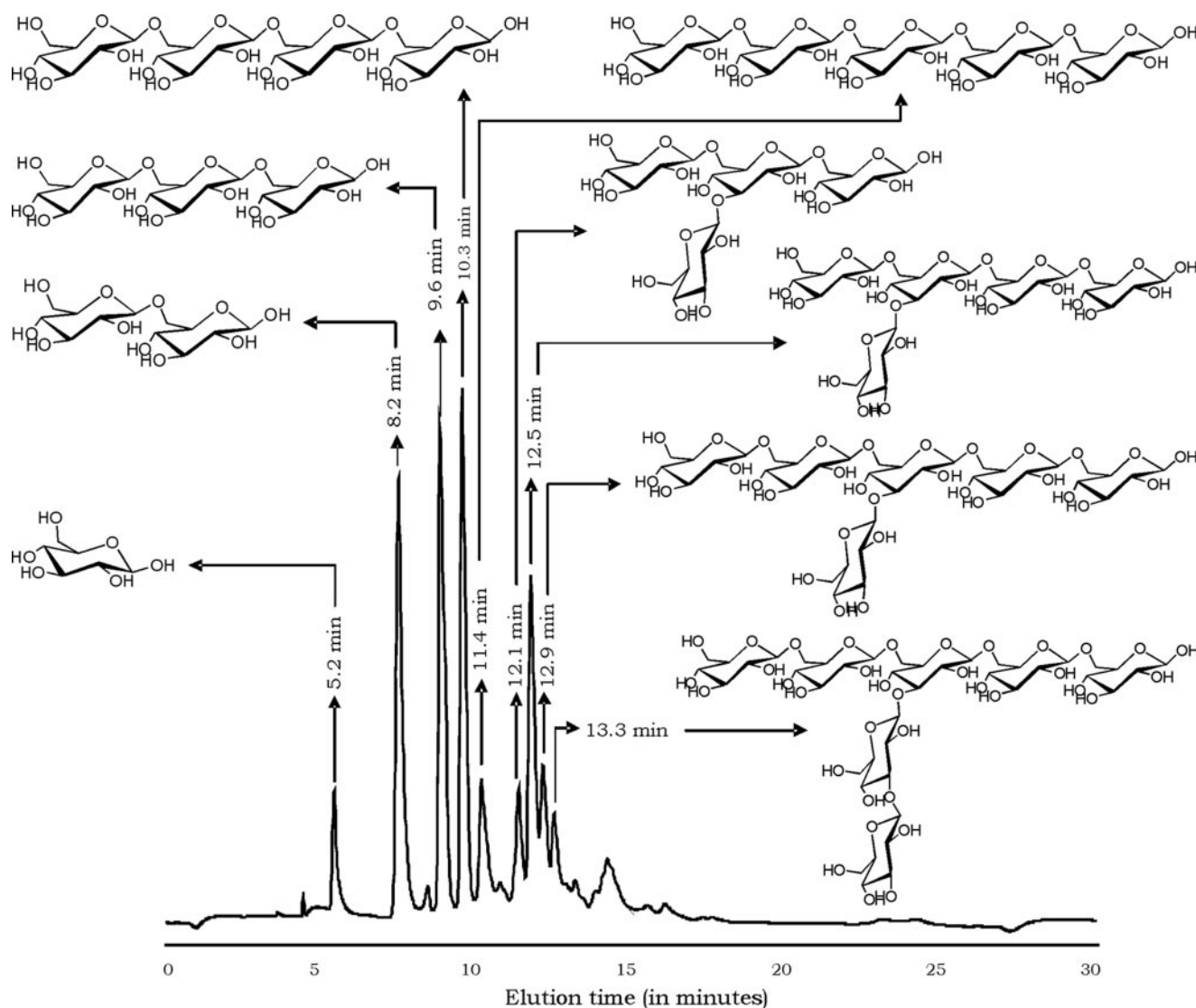


FIGURE 3. Structures of the linear and branched oligomers obtained upon degradation of the 23.6 min peak material with endo- β -(1,6)-glucanase treatment deduced after the characterization of the individual peaks by MALDI-TOF MS, GC-MS, and NMR analyses.

ther, MALDI-TOF analyses confirmed the presence of the oligomers with a degree of polymerization (DP) of 4 and 5 for the peaks with retention times of 10.3 and 11.4 min (m/z ($M + Na^+$) = 689.23 and 851.33, respectively) (since the 2,5-dihydroxybenzoic acid matrix used was a limiting factor for the analysis of the m/z below 500). Oligosaccharides eluted between 12.1 and 13.3 min had m/z ($M + Na^+$) of 689.31, 851.30, 1013.36, and 1175.42, respectively, corresponding to a DP of 4–7. Methylation followed by GC-MS analyses of the oligosaccharides migrating between 12.1 and 13.3 min was characterized by the appearance of 2,3,4,6-Me₄-Glc and 2,4-Me₂-Glc signals, in addition to the major 2,3,4-Me₃-Glc ((1,6)-linked Glc) species, confirming their branching. The appearance of 2,4,6-Me₃-Glc ((1,3)-linked) in the 13.3 min fraction corresponded to the DP 7. NMR analyses further confirmed that peaks migrating between 12.1 and 13.3 min were branched β -(1,6)-glucan-oligosaccharides. Based on these data, possible structures were assigned to the different oligosaccharides resulting from the endo- β -(1,6)-glucanase-cleaved 23.6 min polymer (Fig. 3).

The 1H and ^{13}C NMR chemical shifts and coupling constants obtained for the whole 23.6 min glucan fraction are shown in Table 1. The one-dimensional 1H and two-dimensional 1H , ^{13}C gHSQC spectra exhibited three main signals in the anomeric region (4.722/105.57, 4.534/105.64, and 4.504/105.70 ppm) with areas in a ratio of 1:1:4 from integration in the one-dimensional spectrum (Fig. 4). The 1H and ^{13}C chemical shift analysis and the examination of $^3J_{H,H}$ and $^1J_{C1,H1}$ values allowed the identification of β -glucopyranose residues (40). A NOESY experiment showed a strong correlation between the H1 proton (4.722 ppm) of a glucose residue with the H3 proton (3.733 ppm) of another glucose residue (Fig. 4). Moreover, the H1/C3 correlation between these two glucose residues was observed in the gHMBC experiment (Fig. 4), indicating the following branched sequence: β -Glc_p-(1 \rightarrow 3)- β -Glc_p. This sequence accounts for the downfield chemical shift of the C3 at 87.16 ppm of the 3-substituted glucose residue (Table 1). Besides this, a C6 downfield chemical shift at 71.62 ppm (Table 1) reveals a 3,6-disubstituted glucose residue. Similarly, dipolar interactions between the H1 proton (4.534 ppm) of this disubstituted

TABLE 1

^1H and ^{13}C NMR chemical shifts (ppm) and coupling constants ($J_{\text{H,H}}$ and $^1J_{\text{C,H1}}$, Hz) for the purified 23.6 min peak obtained after β -(1,3)-glucanase digestion of the AI fraction of the *S. cerevisiae* cell wall (wild type)

The AI fraction corresponds to the mean repeating unit: $\rightarrow 6$ - β -Glc-(1 \rightarrow 6)- β -Glc-(1 \rightarrow 6)- β -Glc-(1 \rightarrow 6)- β -Glc-(1 \rightarrow 6)-[β -Glc-(1 \rightarrow 3)]- β -Glc-(1 \rightarrow 6). ND, not determined.

Unit	H1 ($^3J_{1,2}$)	H2 ($^3J_{2,3}$)	H3 ($^3J_{3,4}$)	H4 ($^3J_{4,5}$)	H5 ($^3J_{5,6}$)	H6 ($^2J_{6,6'}$)	H6'
β -Glc-(1 \rightarrow 3)-	4.722 (7.8)	3.340 (8.5)	3.507 (9.4)	3.388 (9.7)	3.479 (ND)	3.717 (ND)	3.904
3,6)- β -Glc-(1 \rightarrow 6)-	4.534 (7.8)	3.511 (8.5)	3.733 (9.4)	3.566 (9.7)	3.644 (ND)	3.872 (ND)	4.212
6)- β -Glc-(1 \rightarrow 6)	4.504 (7.9)	3.310 (8.9)	3.486 (10.0)	3.441 (9.1)	3.612 (5.9)	3.843 (11.1)	4.210

	C1 ($^1J_{\text{C1,H1}}$)	C2	C3	C4	C5	C6
β -Glc-(1 \rightarrow 3)-	105.5 (162.2)	76.14	78.28	72.34	75.77	63.51
3,6)- β -Glc-(1 \rightarrow 6)-	105.64 (ND)	75.53	87.16	70.86	77.39	71.62
6)- β -Glc-(1 \rightarrow 6)	105.70 (162.5)	75.81	78.28	72.28	77.65	71.62

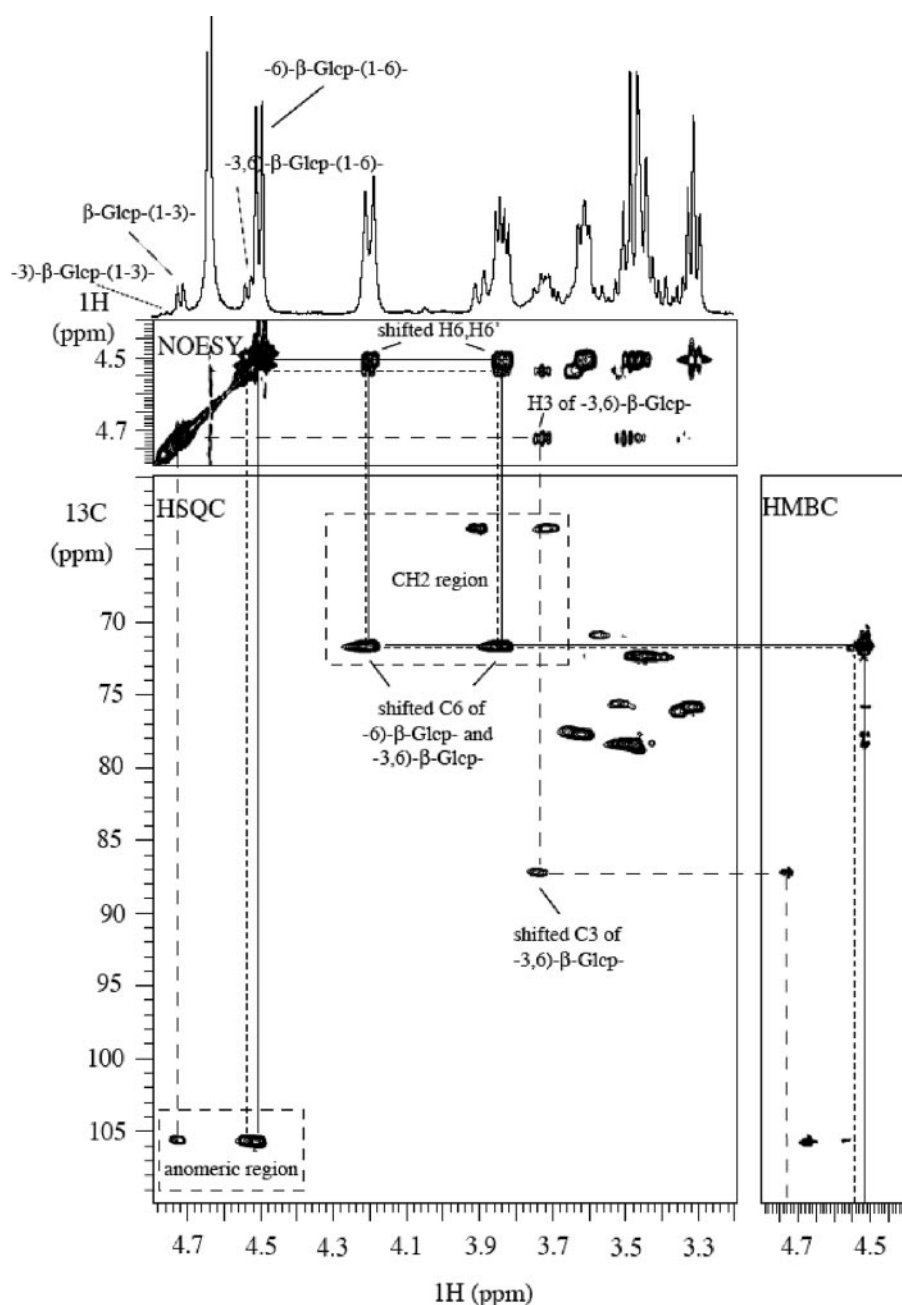


FIGURE 4. NMR spectra of the purified glucan from cell wall. Shown are one-dimensional ^1H on top and two-dimensional ^1H - ^1H NOESY, ^1H - ^{13}C HSQC, and ^1H - ^{13}C HMBC.

glucose residue and H6/H6' protons (3.843–4.210 ppm) of a glucose residue were observed in the NOESY experiment, suggesting the following sequence motif: $\rightarrow 3,6$ - β -Glc-(1 \rightarrow 6)- β -Glc. This linkage was confirmed by the gHMBC experiment with the observation of the H1/C6 correlation between these two glucose residues but also by the downfield chemical shift of C6 of this 6-substituted glucose residue at 71.62 ppm. Strong interactions were also observed in the NOESY experiment between the anomeric proton (4.504 ppm) of this 6-substituted glucose residue and H6/H6' protons (3.843–4.210 ppm) of a glucose residue, corroborated by the presence in the gHMBC experiment of a H1/C6 correlation, indicating the sequence motif $\rightarrow 6$ - β -Glc-(1 \rightarrow 6)- β -Glc, in agreement with the downfield shift at 71.62 ppm of the glucose C6 carbon. These NMR experiments allowed proposing the following mean repeating unit structure: $\rightarrow 6$ - β -Glc-(1 \rightarrow 6)- β -Glc-(1 \rightarrow 6)- β -Glc-(1 \rightarrow 6)-[β -Glc-(1 \rightarrow 3)]- β -Glc-(1 \rightarrow 6).

A branching degree of 0.20 was determined from the ratio of anomeric proton areas of β -Glc-(1 \rightarrow 3) side chain residues to $\rightarrow 6$ - β -Glc-(1 \rightarrow 6) and $\rightarrow 3,6$ - β -Glc-(1 \rightarrow 6) main chain residues in the one-dimensional spectrum. This corresponds on average to a side chain for every five $\rightarrow 6$ - β -Glc-(1 \rightarrow 6)- backbone residues. However, a small anomeric doublet centered at 4.766 ppm, which correlates with H2 at 3.535 ppm and H3 at 3.754 ppm in COSY and RELAY experiments, could be identified as belonging to

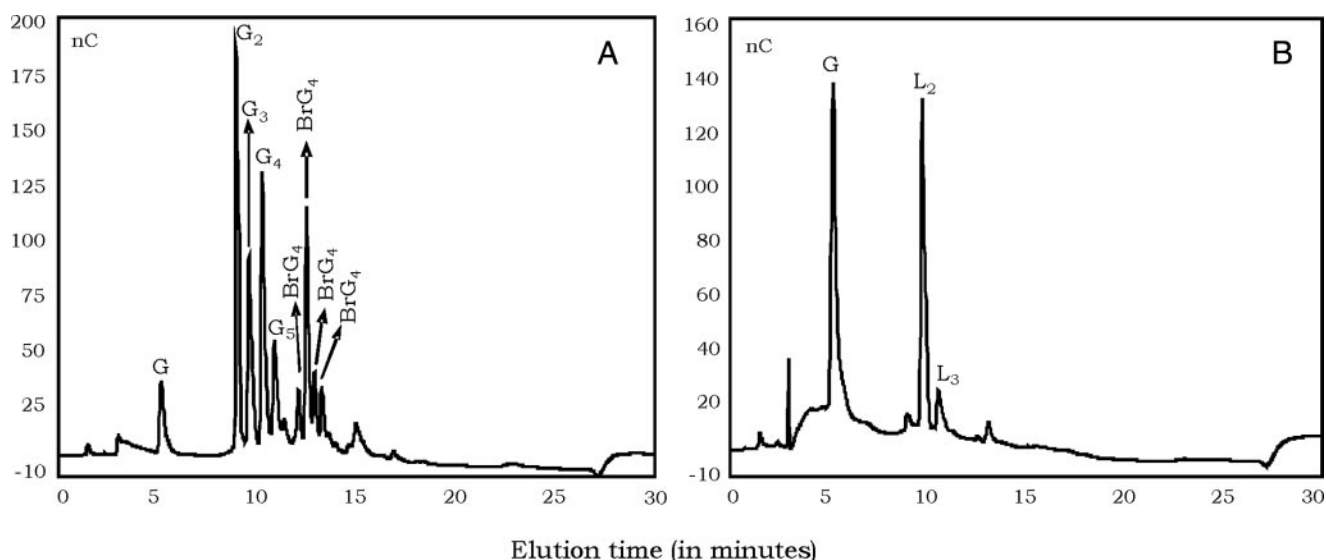


FIGURE 5. **A**, chromatography profiles of the digest obtained by treating directly the AI fraction with endo- β -(1,6)-glucanase (G, glucose; G_n , linear β -(1,6)-linked oligosaccharides of n units; BrG_n , β -(1,3,6)-branched G_n oligomers). **B**, chromatography profiles of the soluble material obtained by the treatment of the endo- β -(1,6)-glucanase undigested material with endo- β -(1,3)-glucanase (G, glucose; L_2 , laminaribiose; L_3 , laminaritriose). Chromatography conditions are as in Fig. 1.

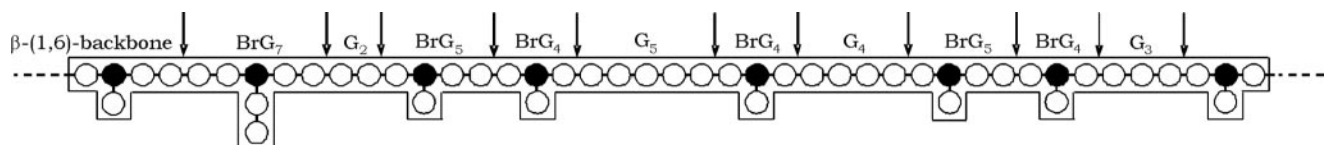


FIGURE 6. **A model of the *S. cerevisiae* cell wall β -(1,6)-glucan.** Empty circles, β -(1,6)-linked glucose units; filled circles, β -(1,3,6)-branched glucose units; gray circles, β -(1,3)-linked side chains. Arrows, possible sites of endo- β -(1,6)-glucanase action on the branched β -(1,6)-glucan.

TABLE 2

Distribution of the radiolabeled glucose after incubation of permeabilized cells or membrane extracts with UDP-[^{14}C]glucose

Cell fractions	Permeabilized cells ^a	Membrane extracts ^a
	nmol glucose incorporated/ h/ μg protein	nmol glucose incorporated/ h/ μg protein
Alkali-soluble fraction	0.28 ± 0.02	0.09 ± 0.03
Alkali-insoluble (AI)	1.17 ± 0.11	0.77 ± 0.03
AI β -(1,3)-glucan	0.75 ± 0.16	0.63 ± 0.04
AI β -(1,6)-glucan (23.6 min peak)	0.24 ± 0.04	0

^a Values were obtained from an average of five experiments, as determined by using a liquid scintillation counter (incubations were performed with a reaction mixture containing UDP-glucose (0.5 mM containing UDP-[^{14}C]glucose), Tris-HCl buffer (50 mM, pH 7.0), ATP (0.2 mM), GTP- γ S (20 μM), MgCl_2 (0.2 mM), EDTA (10 mM), and *N*-acetylglucosamine (20 mM) for 60 min at room temperature in 67 μl).

-3)- β -Glc p -(1 \rightarrow 3)- residues (41). A mean side chain length of 1.1 was thus calculated from the ratio of anomeric proton areas of -3)- β -Glc p -(1 \rightarrow 3)- and β -Glc p -(1 \rightarrow 3) to β -Glc p -(1 \rightarrow 3) side chain residues in the one-dimensional spectrum (Fig. 4).

When the sequence of enzymatic treatment was reversed (endo- β -(1,6)-glucanase followed by treatment of the undigested material with the endo- β -(1,3)-glucanase), the endo- β -(1,6)-glucanase-solubilized AI fraction products showed an HPLC profile similar to the one obtained when the 23.6 min peak was degraded with endo- β -(1,6)-glucanase (Fig. 5A). Upon treatment of the endo- β -(1,6)-glucanase undigested material with endo- β -(1,3)-glucanase, only glucose, laminaribiose, and laminaritriose were released (Fig. 5B). This result showed that β -(1,6)-glucan occupies a terminal position in the AI fraction that is directly accessible to β -(1,6)-glucanase.

Periodate oxidation (disrupting only the β -(1,6)-linked polymers) followed by Smith degradation of the 23.6 min polymer (obtained after gel permeation chromatography of the β -(1,3)-glucanase-solubilized AI fraction) released about 20% of glucose and laminaribiose in a ratio of 10:1, indicating that the side chains were of 1–2 glucose residues, in accordance with the NMR and GC-MS observations. Similar periodic acid/acetic acid treatment of the intact AI fraction solubilized 4% of the total glucan, releasing only glucose and laminaribiose. These quantitative data are in agreement with the overall amount of β -(1,6)-linked glucan found on the AI fraction that is about one-quarter of the total glucan. Taken together, these results indicate the average repeating unit of the β -(1,6)-glucan molecule in the yeast cell wall to be $-(\beta\text{-Glc}p\text{-(1}\rightarrow\text{6)})_{2-6}\text{-}\beta\text{-Glc}p\text{-(1}\rightarrow\text{6)}\text{-}[\beta\text{-Glc}p\text{-(1}\rightarrow\text{3)}]_{1-2}\text{-}\beta\text{-Glc}p\text{-(1}\rightarrow\text{3)}$, which allowed us to propose a β -(1,6)-glucan structure as depicted in Fig. 6.

Neosynthesis of β -Glucan in Permeabilized *S. cerevisiae*—In the presence of UDP-[^{14}C]glucose, permeabilized cells showed incorporation of radioactivity into their cell wall polymer. The total amount of radioactivity incorporated into the whole permeabilized cells and its distribution in the cell wall polysaccharide fractions are given in Table 2. Upon recombinant endo- β -(1,3)-glucanase treatment, there was the solubilization of 75% of the AI fraction-associated radioactivity, indicating that the majority of the radioactivity was incorporated in the β -(1,3)-glucan. Interestingly, the β -(1,3)-glucan synthase activity was slightly higher with the permeabilized cells than with the membrane preparation ($p < 0.05$). The chromatographic profile of the radiolabeled endo- β -(1,3)-glucanase digest (Fig. 7A) was

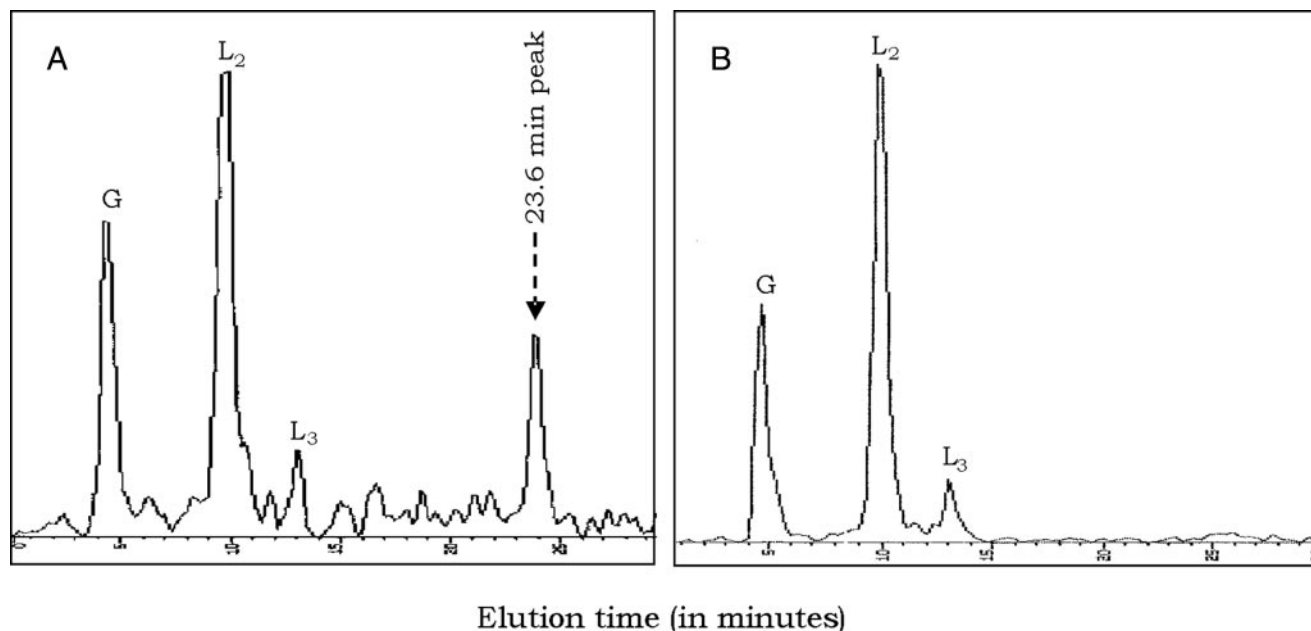


FIGURE 7. Radiomatic profiles of the endo- β -(1,3)-glucanase-solubilized cell wall AI fractions (radiolabeled) obtained with permeabilized cells (A) and a membrane fraction (B). The peaks at 5.1, 10.2, and 13.5 min represent glucose, laminaribiose, and laminaritriose. The broken arrow represents the peak of interest, 23.6 min. Glucose (G), laminaribiose (L_2), and laminaritriose (L_3) peaks are seen in both profiles; however, the 23.6 min peak was absent in the membrane fraction. A slight shift in the retention times of the radiolabeled sugars compared with nonradiolabeled profiles (Fig. 1) is due to the delay in the movement of the solvent from the high performance anion exchange chromatography system to the radioactive detector.

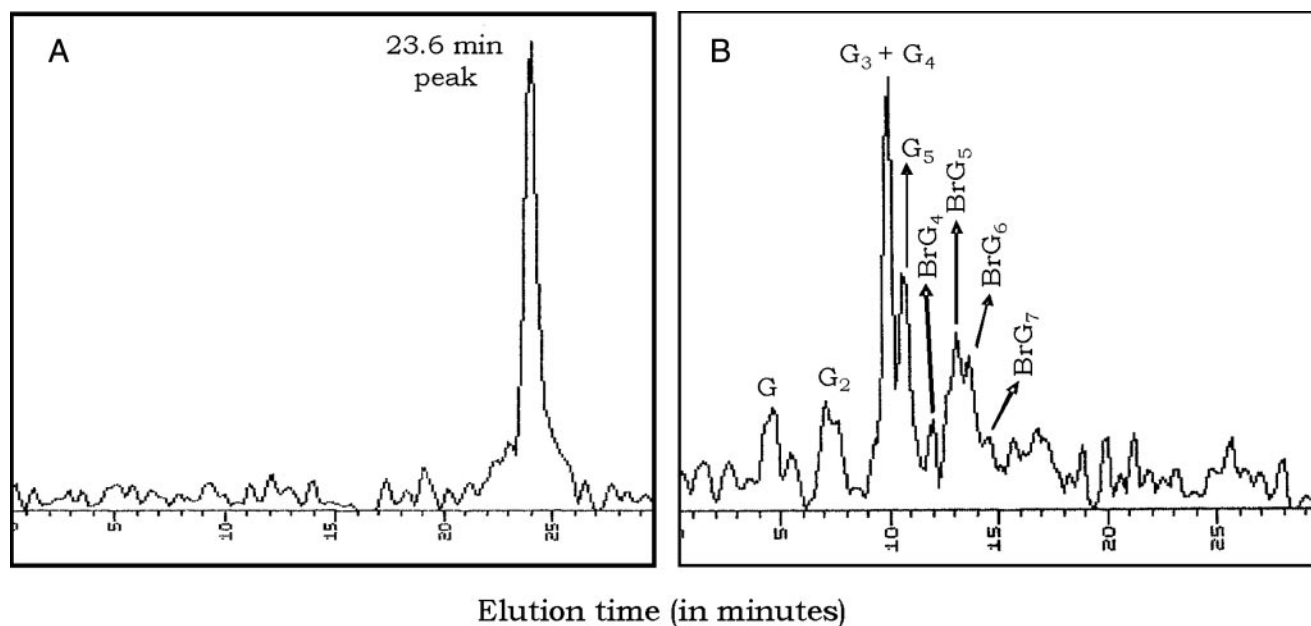


FIGURE 8. Characterization of radiolabeled 23.6 min peak. A, chromatography profile of the 23.6 min peak; B, degradation of the 23.6 min peak following endo- β -(1,6)-glucanase treatment for 24 h (G, glucose; Gn, linear β -(1,6)-linked oligosaccharides of n units; BrGn, β -(1,3,6)-branched Gn oligomers).

identical to the pattern obtained from the digest of the AI fraction of the nonradiolabeled cell wall (Fig. 1A), since only ^{14}C -labeled glucose, laminaribiose, laminaritriose, and the 23.6 min peaks appeared. This result indicated that in the permeabilized cells, the radioactivity was incorporated both into the β -(1,3)- and β -(1,6)-linked polymers. In contrast, when membrane preparations were used as the enzyme source instead of permeabilized cells, the 23.6 min peak was absent, indicating the lack of β -(1,6)-glucan synthesis, whereas β -(1,3)-glucan synthase was still active (Fig. 7B).

The radiolabeled polymer eluting at 23.6 min, when precipitated from the endo- β -(1,3)-glucanase digest using ethanol, accounted for 25% of the total solubilized material. Similar to the nonradioactive 23.6 min peak, there was complete degradation of ^{14}C -radiolabeled 23.6 min fraction upon endo- β -(1,6)-glucanase treatment (Fig. 8) with a pattern similar to the one obtained after the degradation of 23.6 min polymer obtained from the *S. cerevisiae* cell wall. Although the resolution was lower due to the online set-up of the radiomatic detector following HPLC, the appearance of glucose and β -(1,6)-linked oli-

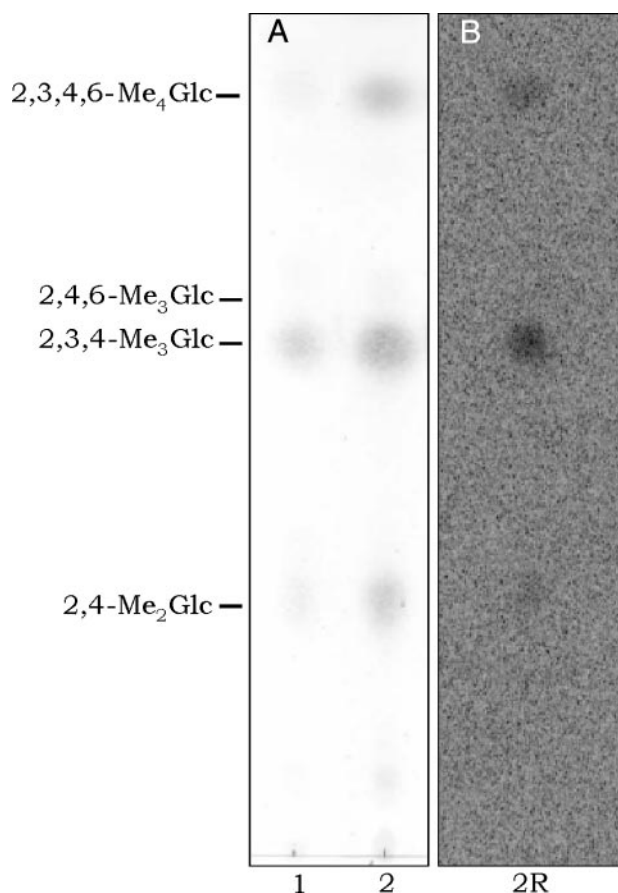


FIGURE 9. TLC profiles of the O-methylated glucoses obtained after methylation, followed by trifluoroacetic hydrolysis of the glucans. Lane 1, 2,3,4-tri-O-methyl-glucose obtained from methylation of pustulan (a glucan with β -(1,6)-linkages; Calbiochem); lanes 2 and 2R, O-methylated products obtained from neosynthesized 23.6 min polymer; samples were revealed either using Orcinol (lane 2) or by PhosphorImager (lane 2R). The lines showing migrations of 2,4,6-tri-O-methyl-glucose, 2,4-di-O-methyl-glucose, and 2,3,4,6-tetra-O-methyl-glucose were deduced from the methylation of curdlan (a kind gift from Dr. Hidemitsu Kobayashi) and laminarin (Sigma) (data not shown). (A, revealed by orcinol reagent; B, revealed by PhosphorImager).

gomers of DP 2–7 in the radioactive profile clearly indicated neosynthesis of β -(1,6)-linkages. The peaks between 4.7 and 11.7 min corresponded to the retention times of glucose and gentiobiose to gentiopentaose, respectively, and accounted for 75% of the radioactivity. The collective peaks between 12.3 and 14.3 min were suggestive of the branched oligosaccharides observed while analyzing nonradiolabeled 23.6 min polymer (Figs. 2B and 8B).

Methylation and trifluoroacetic acid hydrolysis of the 23.6 min polymer followed by the migration of the hydrolysate on the TLC plate showed the presence of mainly 2,3,4-Me₃Glc ((1,6)-linked) along with 2,3,4,6-Me₄Glc (permethylated), and 2,4-Me₂Glc (3,6-branched) species (Fig. 9) in an approximate ratio of 5:1.5:1.0 integrated on the autoradiogram with ImageQuant. These results indicated that neosynthesized 23.6 min polymer showed a structure similar to that of the β -(1,6)-glucan existing in the cell wall, suggesting that the [¹⁴C]glucose incorporated is uniformly distributed on the entire molecule. Further analyses of the Biogel-P2 column-purified oligomers obtained after endo- β -(1,6)-glucanase digestion of the radiolabeled 23.6 min polymer on TLC confirmed incorporation of the

glucose radioactivity over the entire molecule, since the individual separated oligomers were all radiolabeled (supplemental Fig. 1).

There was incorporation of radioactivity into the cell walls of permeabilized $\Delta kre9$ and $kre5-ts2$ mutant cells upon incubation with UDP-[¹⁴C]glucose, although to a lesser extent than the wild type strain. However, chromatography profiles of the endo- β -(1,3)-glucanase-digested AI fraction from these *kre* mutants did not show a 23.6 min peak (supplemental Fig. 2). However, the appearance of radioactive peaks for glucose and β -(1,3)-oligomers in all *kre* mutants were indicative of an active β -(1,3)-glucan synthase. This result showed that the permeabilized cells of the *kre* mutants did not display any β -(1,6)-glucan synthase activity under the experimental conditions, whereas they still showed significant β -(1,3)-glucan synthase activity. Similar analysis was also performed with YG0091 strain in which the gene *ALG5* encoding for UDP-glucose/dolichylphosphate glucosyltransferase has been deleted (26). As is evident from supplemental Fig. 3, both in the nonradioactive and radioactive profiles, there was the presence of the peak corresponding to 23.6 min, and the area was comparable with that observed for wild-type strain (*BY4741*) (Figs. 1A and 7A), ruling out the possibility of dolichylphosphoryl-glucose acting as an intermediate in β -(1,6)-glucan biosynthesis.

Characterization of β -(1,6)-Glucan Synthase Activity—The quantification of the radioactivity incorporated into the 23.6 min peak was used to characterize the physico-chemical parameters of the β -(1,6)-glucan synthase activity. When permeabilized cells were incubated with UDP-[¹⁴C]glucose, there was a linear increase in labeling of the 23.6 min peak up to 60 min. The optimum β -(1,6)-glucan synthase activity was observed at pH 7.0, and there was a 2.5-fold increase in the UDP-[¹⁴C]glucose incorporation when the reaction temperature was shifted from 30 to 25 °C. Maximal activity was observed when the reaction mixture was supplemented with EDTA (radioactivity incorporation increased by 1.5-fold). A double reciprocal plot of the 23.6 min peak area against varying concentrations of UDP-[¹⁴C]glucose indicated an apparent K_m value of 0.62 mM. Maximum substrate incorporated into β -(1,6)-glucan was found to be 0.24 nmol of glucose/h/ μ g of protein in the permeabilized cells.

In contrast to permeabilized cells, membrane fractions did not show β -(1,6)-glucan synthase activity (Table 2). Permeabilized cells were partitioned into cell wall, membrane, and intracellular fractions and assayed individually or in combination to reconstitute β -(1,6)-glucan synthase activity. β -(1,3)-Glucan synthase activity was observed from all individual fractions or in combinations, except for the intracellular fraction. Interestingly, β -(1,3)-glucan synthase activity was higher with the cell wall fraction compared with the membrane preparation (Table 2). However, β -(1,6)-glucan synthase activity was lost as soon as permeabilized cells were disrupted, and combination of fractions did not restore the activity. A similar loss of activity was seen when permeabilized cells were broken either with Fast-prep (MP Biomedicals) or with other instruments, such as MSK Braun cell homogenizer.

DISCUSSION

The *S. cerevisiae* β -(1,6)-glucan structure described here is in agreement with and extends the seminal study published 35 years ago by Manners *et al.* (42). The authors described a water-soluble component resistant to β -(1,3)-glucanase treatment that accounted for 10% of the yeast glucan. Using enzymolysis, periodate oxidation, and methylation analyses, they identified a highly branched β -(1,6)-glucan of about 130–140 glucose residues containing a small proportion (5–10%) of β -(1,3)-glycosidic linkages and 15–25% of the glucose residues on the backbone triply linked at C1, C3, and C6. In comparison, the β -(1,6)-glucan molecule that we characterized has a slightly higher DP than the one isolated earlier by Manners *et al.* (42), since it consists of about 190–200 glucose residues (with 20% branching) instead of the 130–140 reported earlier. Magnelli *et al.* (43) also investigated the structure of the soluble fraction resulting from Zymolyase treatment of an AI fraction of the yeast cell wall. Further treatment of this fraction with a recombinant β -(1,6)-glucanase from *T. harzianum* suggested that it was a highly branched β -(1,6)-glucan. Enzymatic treatment resulted in the release of β -(1,6)-linked linear di- and trisaccharides and β -(1,3,6)-branched tri- and tetrasaccharides. This panel of oligosaccharides is slightly different from the one obtained in the present study. There are several explanations for these discrepancies. (i) The starting material was prepared differently in our study; the cells were first boiled in SDS-ME, a treatment that removes all of the soluble material from the cell wall (44), and the alkali treatment was performed under reducing conditions to avoid peeling of the β -(1,3)-glucan (45). (ii) The endo- β -(1,3)-glucanase (Zymolyase) used by Magnelli *et al.* (43) contained other enzymatic contaminants in addition to the major β -(1,3)-glucanase activity in contrast to the recombinant endo- β -(1,3)-glucanase used in our study. The present study and the earlier ones emphasized the need to use exclusively recombinant glycosyl-hydrolases in the analysis of complex cell wall structures. Three recombinant glucanases are available and can be used successfully to identify cell wall polysaccharide structures. Endo- β -(1,6)- and endo- β -(1,3)-glucanases are capable of degrading only linear structures, and branch points are resistant to their action. LamA (27) (recombinant endo- β -(1,3)-glucanase from *T. neapolitana*) used in the present study releases glucose, laminaribiose, and laminaritriose, whereas Quantazyme (another recombinant endo- β -(1,3)-glucanase from *Oerskovia xanthineolytica*) releases laminaripentaose and laminaritetraose from linear β -(1,3)-glucan (curdian).⁴ The unique recombinant endo- β -(1,6)-glucanase from *T. harzianum* used to date releases mainly G₂ and G₃ and some G₄ and G₅ from pustulan (28).

An interesting finding from our study is the identity of the HPLC pattern resulting from the β -(1,6)-glucanase treatment of either the intact AI fraction or the 23.6 min peak recovered after β -(1,3)-glucanase treatment. This result showed that the short side chains (one/two glucose units) on the β -(1,6)-glucan backbone do not result from the action of the β -(1,3)-glucanase but are present as the integral structure of the β -(1,6)-glucan.

The structure of neosynthesized β -(1,6)-glucan after 10 min and 1 h of incubation with UDP-[¹⁴C]glucose are identical, although the level of radioactivity incorporated into this polymer after 10 min accounted for 20–25% of the incorporation after 1 h. This suggests that the formation of linear β -(1,6)-linkages and the attachment of these branches is a simultaneous process rather than a sequential phenomenon (data not shown). Schmid *et al.* (46) reported simultaneous elongation and side chain insertion during β -(1,3;1,6)-glucan biosynthesis in *Epicoccum nigrum* strain F19, and Batra *et al.* (47) also observed such activity during branched β -glucan synthesis in *Sclerotium rolfii* Sacc. Such simultaneous elongation and branching activities have also been described in bacteria, which could be the result of a single protein or two proteins. In *Streptococcus pneumoniae*, Lull and co-workers (48), reported selective incorporation of glucose from UDP-glucose into a capsular polysaccharide containing two different β -glucosidic bonds (β -(1,3)- and β -(1,2)-) by a single processive β -glucosyltransferase belonging to glycosyltransferase family 2 (available in the CAZy data base on the World Wide Web), whereas in *Pseudomonas aeruginosa*, biosynthesis of osmoregulated periplasmic branched β -glucans is performed through the cooperative action of two proteins, OpgG and OpgH (49).

The function of the short β -(1,3)-glucan side chains is presently unknown, but they could be anchors for other cell wall components, such as glycosylphosphatidylinositol-anchored cell wall proteins, which are known to be linked to β -(1,6)-glucan (50, 51) or chitin side chains, especially when the cell is under stress (52, 53). This result also confirms the role of this β -(1,6)-glucan as a flexible linker in the cell wall organization (Fig. 6). A similar structure of a β -(1,6)-glucan polymer has been recently found in *C. albicans* following an NMR analysis of the high molecular weight subfraction obtained after Zymolyase treatment of an acid-alkali insoluble glucan (41). The low molecular weight subfraction consisted of glucose and short linear laminarioligosaccharides with a DP ranging from 3 to 5 (the trisaccharide being predominant). The undigested β -(1,6)-glucan of 20 kDa consisted of about half of the acid alkali-insoluble fraction, and the one found in *S. cerevisiae* accounted for about 25% of the cell wall alkali-insoluble fraction (43). In *C. albicans*, this polymer consisted of a β -(1,6)-linked glucan backbone with short β -(1,3)-linked side chains of 2.2 units on average. The degree of branching is slightly lower than that found in *S. cerevisiae*, since it has a value of 0.14, accounting for one glucose unit branched for every seven glucose units (instead of one for every fifth glucose unit in *S. cerevisiae*). A similar finding was noted in the analysis of Herrero *et al.* (8). Iorio *et al.* (41) have drawn the β -(1,6)-glucan backbone structure with short linear β -(1,3)-glucan side chains. However, in both *C. albicans* and *S. cerevisiae*, the chemical data are lacking in support of this conclusion, and the β -(1,3)-glucan can also be located either at the reducing or nonreducing ends of the β -(1,6)-glucan.

In our experimental conditions, we were not able to produce a β -(1,6)-glucan polymer using membrane preparations. This is in contrast with the previous reports of Vink *et al.* (17) and Lopez-Romero and Ruiz-Herrera (16). However, in these two studies, the reaction product has never been characterized

⁴ T. Fontaine, unpublished data.

chemically, in contrast to this report. In the study by Vink *et al.* (17), the product of the reaction was quantified through a positive reactivity with a polyclonal antibody raised against β -(1,6)-glucan oligosaccharides (with an average DP of 15) conjugated to bovine serum albumin. Moreover, the specific β -(1,6)-glucan synthetic activity computed from the study of Vink *et al.* (17) is 25 times lower than the one obtained in the present study using permeabilized yeasts. In the study by Lopez-Romero and Ruiz-Herrera (16), the β -(1,6)-linked product synthesized from UDP-glucose by membrane preparations was a disaccharide that, upon chromatography, had a retention time similar to gentiobiose. This disaccharide was released from the neosynthesized β -(1,3)-glucan upon an exo- β -(1,3)-glucanase treatment, indicating its presence at the reducing end of the β -(1,3)-glucan molecule, thus suggesting this to be entirely different from the β -(1,6)-glucan molecule synthesized in the permeabilized yeast cells. In addition, the total amount of radioactivity quantified for this gentiobiose molecule accounts for only 0.6% of the total radioactivity incorporated from UDP-glucose with a membrane preparation. It is unknown to date whether the inhibitory effect of the cell breakage is due to the solubilization of a co-factor that is lost during membrane recovery by centrifugation or the disruption of protein complexes, the integrity of which could be essential for such catalytic activity.

KRE genes have been repeatedly shown to be involved in β -(1,6)-glucan synthesis. Among all *KRE* genes, *KRE5*, -6, and -9 seem the most relevant proteins to be involved in the catalysis of β -(1,6)-glucan synthesis, since their deletion resulted in the lowest amount of β -(1,6)-glucan in the yeast cell wall. There is, however, no biochemical evidence that any of these Kre proteins form the catalytic subunit of the β -(1,6)-glucan synthase. Some arguments do exist that suggest the possibility that Kre5p could be part of the β -(1,6)-glucan biosynthetic machinery. (i) β -(1,6)-Glucans are almost totally absent from a *kre5* mutant. In the *kre5-ts2* mutant, the speck at 23.6 min in Fig. 1C accounting for 2% of β -(1,6)-glucan corresponds to the contribution from the *kre5-ts2* cells in the preinoculum that were not grown at restrictive temperature. (ii) Kre5p displays significant similarity with UDP-glucose glycosyltransferases and contains a highly conserved C terminus with a UDP-glucose putative binding site (7, 8). Other arguments are, however, against the participation of Kre5p to the enzymatic catalysis of β -(1,6)-glucans. (i) Kre5p is localized in the endoplasmic reticulum, since it contains an HDEL endoplasmic reticulum retention signal, whereas synthesis is supposed to occur at the plasma membrane; (ii) in *C. albicans*, a *kre5/kre5* mutant contains one-fourth of WT levels of β -(1,6)-glucan (8), indicating that Kre5p is not the catalytic subunit. Nor does it seem that Kre6p is directly involved in the synthesis of this polymer, since β -(1,6)-glucan is still found in the cell wall of the Δ *kre6* mutant (~30% in our study (data not shown) and ~50% as observed by Roemer and Bussey (54), in comparison with wild-type strain). In addition, an orthologous *KRE6* gene is present in *Aspergillus fumigatus* that does not contain β -(1,6)-glucans in its cell wall. Kre9p remains the most probable candidate to be involved in the biochemical synthesis of β -(1,6)-glucans, since it is putatively located in the cell wall (11) and is essential in *C. glabrata*

and in *S. cerevisiae*, where the double disruption of the two paralogs, *KRE9* and *KNH1*, is lethal (10, 55).

The absence of activation by GTP γ S suggests that the β -(1,6)-glucan pathway is not regulated by a GTP-activated protein similar to Rho1p that regulates β -(1,3)-glucan synthesis. Although the biosynthetic pathways of both β -(1,3)- and β -(1,6)-glucans are differently regulated, data presented here and in other studies show that the synthesis of these two glucan polymers seem coordinated. Reduction of β -(1,3)-glucan either seen in different mutants or consecutively to the addition of β -(1,3)-glucan synthase inhibitors is associated to a diminishment of the β -(1,6)-glucan content of the cell wall (54, 56, 57). These results suggested that β -(1,6)-glucan synthesis anchored neosynthesized β -(1,6)-glucan to β -(1,3)-glucan. Similarly, cell wall extracts or permeabilized cells display a higher β -(1,3)-glucan synthase activity (Table 2) than membrane preparations that have been always used to date as a source of β -(1,3)-glucan synthase activity. These results suggest that a polysaccharide anchor molecule may also be essential to the β -(1,3)-glucan organization in the cell wall. Such anchors that have been identified in plant polysaccharide synthesis (58) will now be searched among fungi.

Acknowledgments—We thank Dr. Bernadette Coddeville (Laboratoire de Chimie Biologique, Université des Sciences et Technologies de Lille, France) for MALDI-TOF MS analyses.

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